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Manipulating RyR2 Ca^{2+} Signalling in Cardiomyocyte Networks using Morpholino Oligonucleotides
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Pharmacologic and gene-based approaches to normalise excitation-contraction coupling (ECC) in heart disease often provoke compensatory adaptations. We proposed that such (mal)adaptation re-configures homeostatic Ca^{2+} signalling network to a new state that may directly contribute to disease pathogenesis (George et al., AJP 2012). We sought to investigate approaches that could modulate disease-linked Ca^{2+} release abnormalities without perturbing homeostatic Ca^{2+} signalling. In this proof-of-concept study we investigated the utility of a morpholino oligonucleotide targeted to exon1 of RyR2 (rMO) to modify Ca^{2+} release in HL-1 cardiomyocyte networks. Imaging of fluorescein-labelled MOs revealed a homogeneous time- and concentration-dependent accumulation of MO in the cytoplasm of >80% HL-1 cardiomyocytes. The efficacy of rMO-mediated protein knock-down was assessed using a construct encoding RyR2 exon 1 (bases 1-25) fused to the N-terminus of eGFP. The use of a control MO (cMO), that has no homology to known mammalian RNAs, and the expression of eGFP minus the RyR2 exon 1 confirmed the specificity of rMO effects. Incubation of HL-1 syncytia with rMO (24-72h, 1-10uM) did not alter RyR2 mRNA abundance and spontaneous Ca^{2+} oscillations and intercellular synchronisation of Ca^{2+} release remained unchanged. Despite near-identical basal Ca^{2+} oscillations in rMO- and cMO-treated HL-1 populations, caffeine-evoked Ca^{2+} transients were modulated by rMO in a concentration- and time-dependent manner. We expanded this study to determine the effects of rMO in human ES-derived cardiomyocytes over a longer time period (21 days). Our data support the development of MO-based methods to modulate discrete aspects of Ca^{2+} signalling in cardiac disease.

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Cardiac Hypertrophy Associated with Impaired Regulation of Type2 Ryanodine Receptor Calcium Channel by Calmodulin and S100A1
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The cardiac ryanodine receptor ion channel (RyR2) is inhibited *in vitro* by calmodulin (CaM). Simultaneous substitution of three amino acid residues in the CaM binding domain (W3587A/L3591D/F3603A, $RyR2^{ADA}$) leads to loss of CaM inhibition at submicromolar (diastolic) and micromolar (systolic) Ca^{2+} concentrations *in vitro* and cardiac hypertrophy and heart failure in mice. To address whether elimination of CaM inhibition at diastolic or systolic Ca^{2+} causes cardiac hypertrophy, a second mutant mouse was prepared with a single amino acid substitution (L3591D, $RyR2^{D}$) in the CaM binding domain. In single channel measurements, $RyR2^{D}$ lost CaM inhibition at diastolic but not systolic Ca^{2+}, and lost inhibition by the small Ca^{2+} binding protein S100A1 at both diastolic and systolic Ca^{2+}. In contrast to $Ryr2^{ADA/ADA}$ mice, $Ryr2^{D/D}$ mice had a normal lifespan and cardiac contractility. In 6-month old $Ryr2^{D/D}$ mice, heart-to-body weight ratio increased by 8% with a two-fold upregulation of atrial natriuretic peptide mRNA levels and a 40% decrease in RyR2 content. Differences between mutant and wild-type mice were more prominent in mice subjected to 4 weeks pressure overload using transverse aortic constriction. The results contrast those of $Ryr2^{ADA/ADA}$ mice that have an impaired cardiac contractile performance and die at ~2 weeks after birth, and suggest that CaM inhibition of RyR2 at systolic Ca^{2+} is important for maintaining normal cardiac function. Supported by NIH, AHA and NSF.

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The CPVT-Associated RyR2 Mutation G230C reduces the Threshold for Store Overload-Induced Ca Release (SOICR)
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Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a life-threatening arrhythmia. Many congenital mutations in both the cardiac ryanodine receptor (RyR2) and calsequestrin (CASQ2) are known to be responsible for this disorder. It is now well established that CPVT is caused by delayed afterdepolarizations (DADs)-induced triggered activities, and that DADs are caused by spontaneous sarcoplasmic reticulum (SR) Ca release during Ca overload, a process also known as store overload induced Ca release (SOICR). A large body of evidence indicates that CPVT-causing RyR2 and CASQ2 mutations enhance the propensity for SOICR and DADs by increasing the response of RyR2 to SR luminal Ca. Recently, Marks and colleagues reported that a CPVT RyR2 mutation G230C increases the cytosolic Ca sensitivity (only after PKA phosphorylation) of single RyR2 channels in lipid bilayers, but has no effect on the luminal Ca sensitivity of the channel. These observations have led to the conclusion that SOICR is not involved in the disease mechanism of the RyR2-G230C mutation. However, the cellular impact of this mutation on SOICR has yet to be determined. To this end, we generated stable, inducible HEK293 cell lines expressing RyR2-WT and the RyR2-G230C mutant. We induced SOICR in these cells by elevating extracellular Ca, and found that the RyR2-G230C mutation markedly enhances the propensity for SOICR. Further, we employed single cell luminal Ca imaging to monitor the luminal Ca dynamics in RyR2-WT- and G230C-expressing cells during store Ca overload. We found that the G230C mutation significantly reduces the luminal Ca level at which spontaneous Ca release occurs (i.e. the SOICR threshold). Therefore, these results and those of previous studies demonstrate that reduced SOICR threshold is a common defect of CPVT-associated RyR2 mutations.

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The L433P Arrhythmia-Linked Mutation Disrupts Amino-Terminus Oligomerisation of the Human Cardiac Ryanodine Receptor
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The type 2 ryanodine receptor (RyR2) mediates calcium release from the sarcoplasmic reticulum of cardiomyocytes. RyR2 mutations found in three clusters including the amino-terminus are associated with arrhythmogenic cardiac disease. Arrhythmia-linked mutations are proposed to disrupt interactions between discrete functional domains within the RyR2 tetramer, resulting in abnormal channel gating. Recently, we presented evidence that the RyR2 N-terminus self-associates into a tetrameric form, which stabilises the closed channel conformation.
Here, we report that the arrhythmia-associated L433P mutation affects oligomerisation of the RyR2 N-terminus. Tetramerisation ability was tested by chemical cross-linking experiments of an RyR2 N-terminal fragment (residues 1-906) containing L433P, expressed in HEK293 cells. The mutant fragment displayed reduced ability for tetramerisation versus wild-type. Two additional techniques, the yeast two-hybrid system and co-immunoprecipitation assays, further indicated that the mutant fragment displays reduced self-interaction and reduced binding to wild-type N-terminus. Notably, dantrolene, a drug used to treat the clinical symptoms of malignant hyperthermia and whose target-binding site lies within the RyR N-terminus, was able to partially restore the tetramer in the L433P mutant.
The effect of the L433P mutation was also investigated in the context of the full-length channel. We performed sucrose density gradient centrifugation of CHAPS-solubilised RyR2 to assess the relative stability of tetrameric functional channels. We found that wild-type RyR2 remains a tetramer consistent with an intact functional channel, whereas a substantial proportion of RyR2/L433P channels is dissociated into monomers.
Our findings suggest that disruption of inter-subunit interactions within the N-terminus of mutant RyR2 might contribute to the mechanism by which some of the arrhythmia-associated mutations result in RyR2 channel dysfunction.

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ATP and Caffeine Binding to the Human RyR2 Central Domain Encompassing a CPVT Mutation Cluster
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The sarcoplasmic reticulum (SR) of cardiomyocytes contains the cardiac ryanodine receptor (RyR2) - a calcium release channel that plays a pivotal role in mediating the SR calcium release essential for cardiac excitation-contraction coupling. Discrete clusters of point mutations, that may comprise important regulatory regions within RyR2, have been associated with catecholaminergic polymorphic ventricular tachycardia (CPVT). In response to a physiological trigger, RyR2 bearing CPVT mutations are believed to cause diastolic calcium leak, which can result in a fatal arrhythmia. A RyR2 central domain, which encompasses both a CPVT-associated region and a predicted ATP binding motif, has been expressed as a soluble recombinant protein. The wild-type protein was compared with three constructs each containing a different CPVT mutation (P2328S, F2331S and N2368I). ATP is a physiological activator of RyR2 activity and caffeine has a similar effect. Fluorescence spectroscopy was used to record changes in the intrinsic fluorescence of the single tryptophan residue present within the expressed domain, and we found

evidence for protein conformational changes upon ATP or caffeine binding. We observed a similar binding affinity of the expressed RyR2 central domain for both compounds, with an EC50 ~100μM. The presence of the individual mutations made no apparent difference to the EC50. However, there was an increased initial fluorescence value observed for each of the mutant constructs compared to WT. Analysis of the central domain sequence revealed homology with conserved motifs typical of P-loop kinases. Interestingly, many of the CPVT mutations present in this region are coincident with sites corresponding to the Walker A, Walker B and LID motifs in ATP binding proteins.

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Effect of FKBP12 in the Conformational Dynamics of RyR1

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The ryanodine receptor (RyR) is an intracellular ion channel with an important role in depolarization-induced Ca^{2+} release in excitable cells. For the skeletal muscle isoform RyR1, the open and closed conformations were determined at 10 Å resolution by cryoEM, and their structural analysis suggest that RyR1, with four inner helices forming a helical bundle, has a similar gating mechanism to that of K+ channels. Upon gating, RyR1's large cytoplasmic domain also undergoes conformational changes, underlying a long-range communication pathway between the ion gate and effectors bound more than 130 Å away. One of such effectors is the FK506-binding protein of 12 kDa (FKBP12), which we localized at the interface of the ''handle'' and the ''clamp'' domains of RyR1. At the functional level, and under activating [Ca^{2+}] concentrations, single channel experiments with purified RyR1 show that FKBP12 increased RyR1's Po and redistributed its subconductance states towards higher conductance levels. At the structural level, we found that under sub-activating [Ca^{2+}] concentrations and in the absence of FKBP12, RyR1's cytoplasmic domain appears to be a structural intermediate of the well-defined open- and closed-state RyR1-FKBP12 conformations, whereas the inner helices remain in the closed conformation. Overall, our studies suggest that while [Ca^{2+}] exerts a dominant effect in RyR1's gating, FKBP12 changes the energy landscape of RyR1's structural transitions.

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Stabilization of the Skeletal Muscle Ryanodine Receptor Ion Channel-FKBP12 Complex by the 1,4-Benzothiazepine Derivative S107

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Activation of the skeletal muscle ryanodine receptor (RyR1) complex results in the rapid release of Ca^{2+} from the sarcoplasmic reticulum and muscle contraction. Dissociation of the small FK506-binding protein 12 subunit (FKBP12) increases RyR1 activity and leads to the impairment of muscle function. The 1,4-benzothiazepine derivative JTV519, and more specific derivative S107 (2,3,4,5,-tetrahydro-7-methoxy-4-methyl-1,4-benzothiazepine), are thought to improve skeletal muscle function by stabilizing the RyR1-FKBP12 complex. Here, we report that SR vesicles enriched in RyR1 bound a large number of S107 molecules with micromolar affinity. The effects of S107 and FKBP12 on RyR1 were examined under conditions that altered the redox state of RyR1. In SR vesicles, S107 increased FKBP12 binding to RyR1 in presence of reduced glutathione and the NO-donor NOC12. S107 was without effect in the presence of oxidized glutathione. Addition of 0.15 μM FKBP12 to SR vesicles prevented FKBP12 dissociation, whereas in the presence of oxidized glutathione and NOC12 FKBP12 dissociation was observed in skeletal muscle homogenates that contained 0.43 μM myoplasmic FKBP12, and this dissociation was attenuated by S107. In single channel measurements, pretreatment of FKBP12-depleted RyR1s with S107 in the absence and presence of NOC12 augmented the FKBP12-mediated decrease in channel activity. The data suggest that S107 can reverse the harmful effects of redox active species on SR Ca^{2+} release in skeletal muscle.

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Investigating the Relationship between FKBP Structure and the Ability to Activate RyR Channels

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We have recently demonstrated, both at the single-channel level and in isolated cardiac cells (Galfre et al., (2011) *PLoS ONE*, 7, Issue 2, e31956), that the cardiac RyR (RyR2) is activated by FKBP12 and that this effect is regulated by the antagonistic action of FKBP12.6. We now investigate whether FKBPs can also activate skeletal RyR (RyR1) and examined the relationship between FKBP structure and the ability to activate RyR1 and RyR2. We generated a mutant FKBP12 ($FKBP12_{E31Q/D32N/W59F}$) where the amino acids Glu^{31}, Asp^{32} and Trp^{59} were mutated to the respective residues found in FKBP12.6 (Gln, Asn and Phe). The effects of the wild type and mutant $FKBP12_{E31Q/D32N/W59F}$ proteins on the single-channel gating of RyR1 and RyR2 were then compared. Our results show that FKBP12.6 promotes activation of RyR1 whereas FKBP12 promotes activation of RyR2. The FKBP12 mutant, $FKBP12_{E31Q/D32N/W59F}$ did not alter RyR2 Po (n=5) but caused significant activation of RyR1; for example Po was 0.021 ± 0.008 before and 0.110 ± 0.026 after addition of cytosolic 1 μM $FKBP12_{E31Q/D32N/W59F}$ (SEM; n=7; $^{**}p<0.01$)). The FKBP12 mutant, therefore, no longer behaves like FKBP12 but instead mimics the actions of FKBP12.6 on both RyR isoforms. In conclusion, we demonstrate that FKBP12 and FKBP12.6 specifically activate RyR2 and RyR1, respectively. Our results also suggest that the ability of FKBP12 and FKBP12.6 to activate RyR channels is contained within just three amino acid residues of FKBP12 (Glu^{31}, Asp^{32} and Trp^{59}) and the corresponding residues in FKBP12.6 (Gln^{31}, Asn^{32} and Phe^{59}). These results shed new light on the interactions of FKBPs with RyR channels and will be important for understanding the dual role of FKBP12 and FKBP12.6 as regulators of sarcoplasmic reticulum Ca^{2+}-release in cardiac and skeletal muscle.

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FRET-based Mapping of Regions involved in FKBP12.6 Binding to the Type 1 Ryanodine Receptor

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The type 1 ryanodine receptor (RyR1) is an intracellular calcium channel that plays an integral role in skeletal muscle excitation-contraction coupling. The functional properties of this protein are regulated by numerous associated proteins, including FKBP12, a skeletal muscle protein that stabilizes full RyR1 channel gating. While the binding site of FKBP12 was originally identified at position 2458 of RyR1, subsequent reports have localized additional binding determinants within the N-terminal domain of RyR1. In this study, we used fluorescence resonance energy transfer (FRET) to determine the orientation and proximity of FKBP relative to sites either within the N-terminal domain or near amino acid position 2458. We utilized the cardiac muscle isoform FKBP12.6 (which binds to RyR1 at the same site as FKBP12 but with higher affinity and stability) labeled with the FRET donor Alexa Fluor 488 at either position 14, 44, 49, or 85. Specific binding of each labeled FKBP to RyR was confirmed in permeabilized HEK-293T cells expressing wtRyR1. Weak energy transfer was observed from FKBP12.6 to the FRET acceptor Cy3NTA, which was targeted to poly-histidine ''tags'' placed in the N-terminal domain. This result suggested that the acceptors were not proximal (~90Å away) to FKBP12.6 bound to RyR1. Next, we measured FRET from FKBP12.6 to Cy3NTA targeted to positions 2157, 2341, 2502 and 2777 of RyR1. FKBP12.6 binding to constructs containing His-tags at positions 2157 and 2502 was significantly reduced relative to binding to wtRyR1. In contrast, we detected strong and weak FRET efficiencies from FKBP12.6 to positions 2341 and 2777, respectively. These data suggest that FKBP12.6 binds near amino acid position 2341 but far from the N-terminal domain of RyR1. Supported by NIH grant R01AR059124 (to JDF and TG) and R01HL092097 (to RLC).

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Physiological Role(S) of RyR1 in Smooth Muscle Cells

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Type 1 ryanodine receptor (RyR1) is a key protein involved in the regulation of the intracellular Ca^{2+} concentration in skeletal muscle cells, playing a crucial role in muscle contraction by releasing Ca^{2+} from the sarcoplasmic reticulum after plasma membrane depolarization. Dysregulation of calcium signals due to defects in RyR1 have been associated with a wide range of primary neuromuscular disorders, including Malignant Hyperthermia Susceptibility and a number of congenital myopathies including Core Myopathies, some forms of Centronuclear myopathy and congenital fiber type disproportion. Though RyR1 is preferentially expressed in skeletal muscles recent data has shown that it is also expressed in some areas of the central nervous system, in some cells of the immune system (B-lymphocytes and dendritic cells) and in smooth muscle cells. Thus mutations in *RYR1* (the gene encoding RyR1) may lead to alterations of Ca^{2+} homeostasis not only in skeletal muscle, but also in other tissues expressing this intracellular calcium release channel.